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IN VITRO MASS PROPAGATION OF HERMAPHRODITIC *CARICA PAPAYA* CV. MEIZHONGHONG

KUNLIN WU, SONGJUN ZENG*, ZHILIN CHEN AND JUN DUAN

Key Laboratory of South China Agricultural Plant Genetics and Breeding, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, People's Republic of China
**Corresponding author's e-mail: zengsongjun@scib.ac.cn; Phone: +86-20-37252993*

Abstract

An efficient *In vitro* regeneration system was developed for rapid clonal propagation of hermaphroditic *Carica papaya* cv. Meizhonghong using shoot buds from seedlings and axillary buds from greenhouse and field fruit-bearing plants. Explants from seedlings were more easily disinfected and initiated, and had higher multiplication rates and rooting percentages than explants from mature plants. The highest percentage of contamination-free explants from seedlings was 81.7% using $AgNO_3$ treatment after conventional disinfection, which was higher than the antibiotic treatments. Shoot initiation was achieved in Murashige & Skoog (MS) medium with 0.5 mg l^{-1} 6-benzyladenine (BA) and 40 g l^{-1} sucrose. An average of 6.7 fold proliferation rate per subculture was obtained on MS medium containing 0.25 mg l^{-1} BA and 40 g l^{-1} sucrose after five subcultures. Shoot elongation was induced in MS medium supplemented with 0.25 mg l^{-1} BA and 1.0 mg l^{-1} GA_3 and 40 g l^{-1} sucrose. Cultures were maintained for 20 months (22 subcultures) without any loss in multiplication rate or change in growth habit. The 3/2 MS medium (i.e. with 1.5 times the macro-elements of MS) with 500 mg l^{-1} activated charcoal and 5 g l^{-1} sucrose was optimum for root development. In the greenhouse, bags with sand (1/3 v) over a mixture of sieved peat and vermiculite (1:1 v/v) were used and 87% of plants survived. About 100,000 plantlets were produced successfully for field growth within three years. Compared to mother plants, the *In vitro* raised plants were all hermaphrodites and showed no distinct phenotypic variation. This regeneration system is suitable for mass multiplication of cv. Meizhonghong.

Introduction

Carica papaya is an important fruit crop in the tropical and subtropical regions. The species has three sex types: staminate, pistillate and hermaphrodite (Reuveni *et al.*, 1990; Dinesh *et al.*, 2001). The hermaphroditic cv. Meizhonghong hybrid (cv. Sunrise × cv. Shuizhonghong) has elliptical fruit of higher commercial value in South China than pistillate fruit because of its shape and flavor. The seeds from hermaphrodites produce pistillate, hermaphroditic and some staminate adult plants. Mature papaya plants are very difficult to survive in transplanting. In commercial production, two seedlings are usually planted together, and when they grow their sexes are determined and only hermaphrodites are kept. There are cases in which none of the two seedlings are hermaphrodites. Conventional asexual propagation techniques, such as grafting and rooting of cuttings (Allan, 1964; Soomark & Tai, 1975), have not resulted in efficient mass propagation method for hermaphroditic papaya. Clonal propagation of selected hermaphroditic plants of papaya could offer a valuable alternative in field planting.

There have been a number of reports on tissue culture of papaya, by callus or somatic embryogenesis (DeBruijne *et al.*, 1974; Mehdi, 1975; Yie & Liaw, 1977; Arora, 1978; Litz & Conover, 1982; Jordan *et al.*, 1983; Pandey & Rajeevan, 1983; Khatton & Suiltana;1994; Anandan *et al*., 2012) and shoot culture (Litz & Conover, 1977, 1978a, 1978b, 1981; Rajeevan & Pandey, 1983, 1986; Drew & Smith, 1986; De Winaar, 1988; Drew, 1988; Islam *et al.*, 1993; Manshardt & Drew, 1998). Some have been successful, but commercial *In vitro* mass propagation has not been reported because of many problems in micropropagation as follows:

(1) One major obstacle to successful sterile culture of papaya has been the extremely high rate of microbial contamination by endogenous bacteria from mature plants from the field (Litz & Conover, 1978a, 1978b, 1981; Drew & Smith, 1986; Rajeevan & Pandey, 1986; De Winnaar, 1988; Mondal *et al.*, 1990; Reuveni *et al.*, 1990; Thomas *et al.*, 2007; Thomas & Kumari, 2010), as other plant Gerbera jamesonii (Altaf, *et al*., 2009).
(2) The proliferation rate is low and apical dominance in shoots could not be established during subculturing (Litz & Conover, 1981; Rajeevan & Pandey, 1986).
(3). Normal roots production and acclimatization are difficult for establishing papaya micropropagules (De Winnaar, 1988; Drew & Miller, 1989).

We were unable to use whichever of the reported techniques for *In vitro* mass propagation of the important cv. Meizhonghong because of one or more above-mentioned problems in procedure of tissue culture. The aim of this study was to improve the different stages of *In vitro* propagation of papaya to allow commercial production.

Materials and Methods

Plant material and explant disinfection: Two kinds of plant materials were used. One of the materials was shoot buds of length 3–5 mm were collected from greenhouse seedlings 2–3 months old, Afterwards, seedlings were planted in the field to observe characteristics and determine sex. Only cultures of seedlings from hermaphrodites were used in advanced treatments i.e., multiplication and rooting. The other kind of plant materials was apical buds from fruit-bearing 1 year-old hermaphrodite plants growing in greenhouse and field were removed to promote axillary bud growth: axillary buds 5mm long were collected.

Explants were disinfected with 5 methods: (1) Conventional Method: The explants had leaves removed, and were washed in running tap water for 1 h, immersed in 70% ethanol for 60 s, then in 0.1% (w/v) $HgCl_2$ solution with 0.1% Tween 20® for 10 min., and rinsed 5 times with sterile distilled water. (2) After treatment with the Conventional Method, the explants were dipped in 0.1% (w/v) $AgNO_3$ solution with continual agitation for 5 min and rinsed five times with sterile distilled water. (3) Before the explants were used for culture, mother plants were sprayed with Gentamycin solution (1000 mg l^{-1}) every 24 h for 7 d, then explants were treated with the Conventional Method and shaken at 100 rpm in Gentamycin solution (500 mg l^{-1}) for 3 h on a horizontal shaker. (4) Mother plants were sprayed with Rifampicin (RIF) solution 600 mg l^{-1} and explants were treated with the Conventional Method, then shaken at 100 rpm in RIF (300 mg l^{-1}) for 24 h. (5) Mother plants were sprayed with Gentamycin solution (1000 mg l^{-1}) and explants were treated with the Conventional Method then shaken at 100 rpm in RIF solution (300 mg l^{-1}).

Shoot initiation and multiplication: The sterilized explants were cultured on Murashige & Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 6-benzyladenine (BA; 0.0, 0.1, 0.25, 0.5 and 1.0 mg l^{-1}) and 40 g l^{-1} sucrose for shoot initiation. After 4 weeks of culture, contamination-free explants were transferred to their respective same media for another four weeks, then the regenerated adventitious buds from explants were transferred to the same media for proliferation and growth. After several subcultures, the adventitious buds regenerated from seedlings were cultured on MS with 0.25 mg l^{-1} BA at low temperature (15°C) to slow down the proliferation and growth. When they were determined to be hermaphrodites with good qualities, they were cultured in normal conditions (26 ± 2°C) and multiplied on a large scale.

After a few subcultures, adventitious buds were briefly inoculated in multiplication media MS with 0.25 mg l^{-1} BA, which is not suitable for continuing multiplication and rooting. For those which required a phase for elongation culture, MS containing 0.25 mg l^{-1} BA with different gibberellic acid (GA_3) concentrations (0.0, 0.15, 0.3, 0.5 or 1.0 mg l^{-1}) or MS only containing 0.15 or 0.3 mg l^{-1} GA_3 were assessed**.**

Rooting: Five individual elongated shoots (3–5 cm) from which 1–2 mm of stem base was removed were cultured in a culture container containing MS medium with different concentrations of plant growth regulators or their combinations. The root induction and development was on media supplemented with different indole-3-butyric acid (IBA) concentrations (0.0, 0.25, 0.5, 1.0, 2.0 or 3.0 mg l^{-1}), or napthaleneacetic acid (NAA; 0.0, 0.1, 0.2 or 0.5 mg l^{-1}), or media with activated charcoal (10, 30, 60, 100, 250 or 500 mg l^{-1}), or MS containing 1.0 mg l^{-1} IBA, 60 mg l^{-1} activated charcoal and choline chloride (Sigma**,** 5, 10 or 20 mg l^{-1}).

Two other alternative treatments for root induction were tested. The first one consisted of IBA (0.0, 0.25, 0.5, 1.0, 2.0 and 3.0 mg l^{-1}) in MS medium for 0–6 d. For the second alternative treatment, the base of the shoots were dipped in IBA (200, 500, 1000 and 2000 mg l^{-1}) for 10, 15 or 20 s. Subsequently, shoots from both treatments were transferred to 3/2MS, MS or 1/2MS medium with sucrose (5, 10, 20 or 30 mg l^{-1}) and riboflavin (0.0, 0.5, 1.0, 2.0 or 3.0 mg l^{-1}) or activated charcoal (100, 300, 500, 1000 or 5000 mg l^{-1}) for 3 weeks. Rooting percentages were recorded four weeks after culturing under the different conditions.

Transfer of *In vitro* propagated plants to soil: Micropropagated plantlets with well-developed root systems were transferred to natural conditions for acclimation for 7 d before being transferred to planting bags. Then the plantlets were taken out and the medium washed off the roots with running tap water. The plantlets were transplanted to 10-cm diameter polyethylene planting bags with holes for draining water, containing a mixture of sand, sieved peat and vermiculite (1:1:1; v/v) or 1/3 v sand over a mixture of sieved peat and vermiculite (1:1 v/v) in the greenhouse. The bags were placed in planting beds and covered with polyethylene pellicle to maintain a relative humidity of 80–90% under periodic misting for the first week. Polythene covers were removed gradually within two weeks after transplanting; the plantlets were fertilized weekly with 2g l^{-1} KH_2PO_4. After four weeks, the percentage of plantlet survival was recorded and plantlets were transferred to 25-cm diameter polyethylene planting bags in an acclimation greenhouse for another four weeks. Finally, the 40–50 cm high plantlets were planted in the field.

Culture conditions and statistical analysis: The media was adjusted to pH 5.8 with 1 mol l^{-1} KOH and 1 mol l^{-1} HCl before autoclaving at 121°C for 20 min., at 1.06 kg cm^{-2}. GA_3 and RIF were filter-sterilized. The cultures were incubated at 26 ± 2°C with 16 h/8 h of day/night under a cool white fluorescent light delivering approximately 45 μmol m^{-2} s^{-1} (Zeng *et al*., 2008).

All experiments were analyzed as completely randomized designs. Each experiment was repeated three times and consisted of five explants per flask and 10 replicate culture flasks. The data were analyzed using one-way ANOVA, and the level of Least Significance Difference (LSD) was determined using Duncan's multiple range test at $p<0.05$ for comparing means of the treatments (Wilkinson, 1986).

Results

Explant disinfection: There were significant differences in contamination frequency from different explants and different disinfection methods. There were more contamination-free explants with regeneration capacity from greenhouse seedlings than from mature plants. Only 8.3% of explants were contamination-free from mature field-grown plants with the conventional disinfection method (Table 1). Seedlings treated with $AgNO_3$ had the highest percentage of contamination-free explants (81.7%) (Table 1). Seven days of spraying with Gentamycin (1000 mg l^{-1}) before removing the explants and shaking the buds with 300 mg l^{-1} RIF was the most efficient in reducing contamination of buds from fruit-bearing plants in the greenhouse and field, but was not significantly different to the other antibiotic treatments.

Table 1. Effect of different disinfection methods on the rate of infection-free explants from seedlings and fruit-bearing plants in field or greenhouse.

	Disinfection methods	Uninfected explants (%)		
		Seedlings in greenhouse	Fruit-bearing plants in greenhouse	Fruit-bearing plants in field
1.	Conventional method (70% ethanol +0.1% $HgCl_2$)	66.7 ± 4.2cA	26.7 ± 4.2cB	8.3 ± 3.1cC
2.	Conventional method +$AgNO_3$	81.7 ± 1.7aA	43.3 ± 4.2bB	11.7 ± 3.1bcC
3.	Spraying with Gentamycin and shaking in Gentamycin	68.3 ± 3.1bcA	46.7 ± 3.3abB	16.7 ± 4.2abcC
4.	Spraying with RIF and shaking in RIF	75.0 ± 4.3abcA	48.3 ± 3.1abB	18.3 ± 3.1abcC
5.	Spraying with Gentamycin and shaking in RIF	78.3 ± 4.0abA	55.0 ± 4.3aB	21.7 ± 3.1abC

Each value is the mean ± SE of 60 replications

Values followed by the small different letters within columns or capital letters within rows are significantly different at $p<0.05$

Shoot initiation; Different types of explants and BA concentrations were tried for improving clonal propagation of papaya *In vitro* by increasing proliferation rates. Adventitious shoots derived from adult trees were significantly more difficult to induce than from seedlings, on all media (Table 2). The initial response to culture was swelling and enlargement of the sterilized buds and unfolding of leaves, occurring within 8 d in those from seedlings and 12 d from the fruit-bearing plants on medium supplemented with 0.5 mg l^{-1} BA. The 0.5 mg l^{-1} BA was the most suitable when compared to other concentrations tested for initiation (Table 2). Four weeks after inoculation, contamination-free explants were transferred to the every same media for another four weeks. Adventitious buds were not regenerated on MS medium without BA. Although the highest adventitious regeneration rates were obtained with 1.0 mg l^{-1} BA, prolific calli were at the base of explants and the adventitious buds were very compact with highly shortened internodes.

Table 2. Effect of different concentrations of BA on adventitious shoot production from seedlings and fruit-bearing plants in eight-week establishment culture.

BA concentrations (mg l^{-1})	Mean number of adventitious shoots per explants		Remarks	
	Seedlings	Fruit-bearing plants	Seedling	Fruit-bearing plants
0	0.0 ± 0.0iA	0.0 ± 0.0eA	No callus	No callus
0.10	3.2 ± 0.37gA	1.9 ± 0.31dB	No callus	No callus
0.25	4.3 ± 0.37dA	3.5 ± 0.52cB	No callus	Few calli
0.50	5.3 ± 0.36bA	4.0 ± 0.38bB	Few calli	Few calli
1.00	5.9 ± 0.42aA	5.0 ± 0.57aB	Prolific, calli	Prolific,calli

Each value is the mean ± SE of 60 replications

Values followed by the small different letters within columns or capital letters within rows are significantly different at $p< 0.05$

Multiplication: Subculturing of adventitious buds was necessary after four weeks and at each subsequent four week interval. The multiplication rate gradually increased from two to four subcultures. After five transfers, the mean multiplication rate of adventitious buds was stable; this was higher from seedlings compared to mature plants but not significantly different (Data not shown).

The average multiplication from seedlings was 7.5-fold per subculture on MS media containing 0.5 mg l^{-1} BA and 6.7-fold with 0.25 mg l^{-1} BA (Table 3). However, cultures were very compact with highly shortened internodes and quantities of callus were formed on media with 0.5 mg l^{-1} BA. Such growth presented considerable difficulty for subculturing and rooting and also limited the proportion of shoots suitable for plantlet production. Adventitious buds produced on media with 0.25 mg l^{-1} BA with higher multiplication rate and lower callus could be continuously used for subculture, but were not suitable for rooting. Those cultures were cultured at 15°C on media with 0.25 mg l^{-1} BA to slow multiplication and growth, and were subcultured every eight weeks. After about six months from beginning explant culture, the sex of explants from field-grown seedlings' cut apical shoots was expressed. The hermaphroditic cultures recovered multiplication and growth similarly to the other cultures at normal temperature (26 ± 2°C) (Fig. 1b). Cultures were maintained for > 20 months (22 subcultures) without any decline in multiplication rate or change in growth habit.

Elongation: The shoots were very compact, with shortened internodes and small leaves, when inoculated in multiplication media MS containing 0.25 mg l^{-1} BA and 40 g l^{-1} sucrose, but were not suitable for rooting and required an elongation stage after multiplication. GA_3 induced elongation of papaya shoots *In vitro*, but decreased the shoot proliferation rate (Table 3). Without any plant growth regulators there was poor elongation. If only GA_3 was used, or a high concentration GA_3 in combination with BA in media, then papaya shoots were thin with small leaves and not suitable for rooting. The best elongation was with 3/2 MS basal medium containing 0.25 mg l^{-1} BA, 0.3 mg l^{-1} GA_3 and 40 g l^{-1} sucrose. On this medium, the average height of shoots was 1.54 cm and the shoot proliferation rate was 4.20 (Fig. 1c and Table 3). This medium is suitable for subculture of adventitious bud proliferation in mass commercial production.

Table 3. Effect of different concentrations of GA_3 alone or together with BA on multiplication and elongation of adventitious buds from seedlings.

Plant growth regulators (mg l^{-1})		Multiplication rate	Mean shoot length (cm)
GA_3	BA		
0	0.25	6.7 ± 0.66b	0.53 ± 0.11f
0	0.50	7.4 ± 0.72a	0.44 ± 0.10g
0	0	1.9 ± 0.60h	0.60 ± 0.13f
0.15	0.25	5.4 ± 0.79c	1.02 ± 0.37e
0.30	0.25	4.2 ± 0.67d	1.54 ± 0.27d
0.50	0.25	3.6 ± 0.54e	1.83 ± 0.28c
1.0	0.25	2.3 ± 0.46f	3.01 ± 0.51a
0.15	0	2.3 ± 0.58f	1.88 ± 0.32c
0.30	0	1.5 ± 0.41g	2.05 ± 0.30b

Each value is the mean ± SE of 30 replications
Values followed by the small different letters within columns are significantly different at $p<0.05$

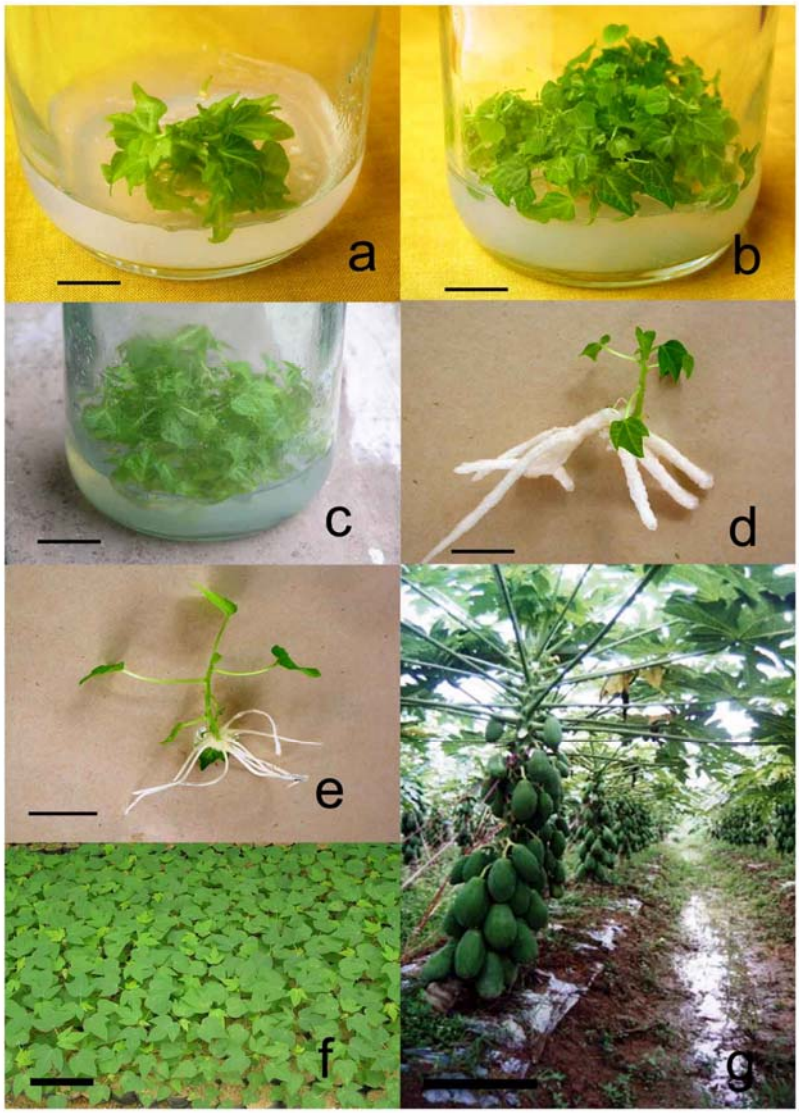

Fig. 1. Plant regeneration of *Carica papaya* cv. Meizhonghong: (a) Adventitious shoot bud induction from shoot on MS medium with 0.5 mg l^{-1} BA (bar = 20 mm). (b) Adventitious bud multiplication on MS medium with 0.25 mg l^{-1} BA (bar = 20 mm). (c) Shoot bud elongation on MS medium with 0.25 mg l^{-1} BA and 0.3 mg l^{-1} GA_3 (bar = 25 mm). (d) Plantlet with thick roots on MS with 1.0 mg l^{-1} IBA, 0.1 mg l^{-1} NAA, 60 mg l^{-1} activated charcoal, 10 mg l^{-1} choline chloric (bar = 10 mm). (e) Plantlet with normal roots on 3/2 MS medium with 500 mg l^{-1} activated charcoal and 5 g l^{-1} sucrose (bar = 10 mm). (f) Surviving plantlets growth 30 d after transplantation (bar = 20 cm). (g) Fruit-bearing plants *In vitro* planting in field (bar = 50 cm).

Rooting: Using adventitious buds of cv. Meizhonghong, it was difficult to obtain both high rooting percentage and good root quality on a medium. The mean rooting percentage of shoots from seedlings was higher (although not significant) than from mature plants; rooting percentage and root quality from subculturing 12–22 adventitious shoots were not obviously different (data not shown). The content of activated charcoal greatly influenced root regeneration, when > 250 mg l^{-1} the regeneration rate of all auxin treatments was obviously lower. With activated charcoal < 60 mg l^{-1}, all auxin treatments produced short and thick roots or roots with few or no lateral branches, or only produced calli. Supplementation of 10 mg l^{-1} choline chloric in medium increased root regeneration. The best medium for root regeneration was MS containing 1.0 mg l^{-1} IBA, 60 mg l^{-1} activated charcoal and 10 mg l^{-1} choline chloric, the rooting percentage was 95.6%, but the regenerated adventitious roots became stout on development culture and were not suitable for transplanting (Fig. 1d).

When shoots were cultured on MS containing 0–3.0 mg l^{-1} IBA for intervals of 0–6 d, and then transferred to 3/2 MS medium with 500 mg l^{-1} activated charcoal and 5 g l^{-1} sucrose, rooting percentage was significantly different to other treatments and root quality also varied with concentration and time of exposure to auxin (Fig. 2). Without IBA in the medium, rooting did not occur. The best treatments for rooting percentage and root quality were from 2-day culture on MS medium with 0.5 mg l^{-1} IBA or 1-day culture with 1.0 mg l^{-1} IBA, before transfer to rooting development media of 3/2 MS medium with 500 mg l^{-1} activated charcoal and 5 g l^{-1} sucrose. These two IBA concentrations produced high rooting percentages of 88% and 90%, respectively, and the regenerated plantlets had numerous lateral branches (Fig. 1e); while those from 2–4 day culture with 1.0 mg l^{-1} IBA or 3-4 day culture with 1.0 mg l^{-1} IBA produced thick roots in this media. With 5–6 days IBA exposure, roots were progressively stouter and thicker, with few laterals. When IBA concentrations > 1.0 mg l^{-1}, the regenerated roots were stout or produced calli.

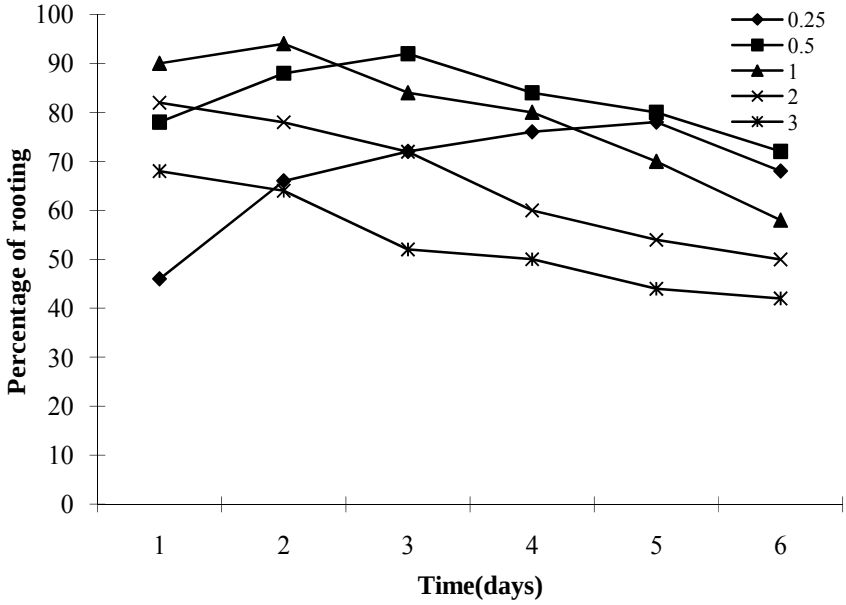

Fig. 2. Effect of IBA concentrations (0.25–3.0 mg l^{-1}) and treatment time on mean rooting percentages of adventitious buds of *Carica papaya* cv. Meizhonghong before transfer to rooting development 3/2 MS medium with 500 mg l^{-1} activated charcoal and 5 g l^{-1} sucrose.

Papaya shoots were dipped in different IBA concentrations (200,500, 1000 or 2000 mg l^{-1}) for 10, 15 or 20 s and then transferred to 3/2 MS containing 10 mg l^{-1} riboflavin or 500 mg l^{-1} activated charcoal to detect any effect of IBA on rooting (Table 4). Shoots transferred to a media containing activated charcoal had finer roots than those with riboflavin. When IBA concentrations < 2000 mg l^{-1}, in addition to 15 s dipping with 500 mg l^{-1} IBA, and were transferred to medium containing 500 mg l^{-1} activated charcoal then rooting percentage was higher but not significantly different to those containing 10 mg l^{-1} riboflavin. When IBA concentration was 2000 mg l^{-1}, the results were reversed; the highest rooting percentage was 92.7% at 15 s of 2000 mg l^{-1} IBA treatment before transfer to 3/2 MS containing 500 mg l^{-1} activated charcoal, but roots were stout. With 10 s treatment of 1000 mg l^{-1} IBA, before transfer to medium with 500 mg l^{-1} activated charcoal, the rooting percentage was 84.7% and the shoots were normal.

Different concentrations MS and sucrose affected rooting development culture. The qualities of roots and shoots on 3/2 MS medium were better than on MS or 1/2 MS medium, with no significant differences for sucrose concentrations of 5, 10, 20 or 30 g l^{-1} (Data not shown).

Hardening and acclimatization: When *In vitro* plantlets of papaya without roots or with thick and stumpy roots were transferred to polyethylene planting bags, only 10–20% of plantlets survived. Plantlets with well-developed roots without prior hardening in natural

conditions also had low rates of survival (30–40%). With *In vitro* hardening of plantlets with well-developed roots transferred to 10 cm diameter polyethylene planting bags of mixed sand, sieved peat and vermiculite, 70% of plantlets survived; in bags with sand (1/3 v) over a mixture of sieved peat and vermiculite (1:1 v/v) 87% plants survived in the greenhouse. Four weeks after transfer, plantlets were transferred to bigger containers (25-cm diameter) and kept in a greenhouse until planted in the field (Fig. 1f). Using the procedure described above, about 100,000 plants were produced and planted successfully in the field within three years. Compared to mother plants, the *In vitro* raised plants were all hermaphrodites and showed no distinct phenotypic variation (Fig. 1g).

Table 4. Effect of different concentrations of IBA and culture dipping time on rooting of elongated shoots.

IBA concentrations (mg l^{-1})	Dipping time (s)	Rooting (%)		Remarks	
		Medium I	Medium II	Medium I	Medium II
0	0	0.0 ± 0.0oA	0.0 ± 0.0oA	□□	□□
200	10	18.0 ± 3.1mnA	13.0 ± 2.8nA	Normal roots	Normal roots
200	15	22.7 ± 2.8 lmA	18.7 ± 2.7mnA	Normal roots	Normal roots
200	20	30.7 ± 3.6 lA	25.3 ± 3.3 lmA	Normal roots	Normal roots
500	10	50.0 ± 3.9jkA	42.7 ± 3.7kA	Normal roots	Normal roots
500	15	65.3 ± 3.3ghA	55.3 ± 3.0ijB	Normal roots	Normal roots
500	20	75.3 ± 3.3defA	68.0 ± 4.1fghA	Normal roots	Normal roots
1000	10	84.7 ± 2.7abcdA	77.3 ± 2.3cdefA	Normal roots	Normal roots
1000	15	92.7 ± 2.0aA	85.3 ± 2.3abcA	Stout roots	Stout roots
1000	20	88.0 ± 2.6abA	88.0 ± 2.3abA	Callus formed	Stout roots
2000	10	80.0 ± 2.9abcdA	83.3 ± 2.6abcdA	Callus formed	Callus formed
2000	15	73.3 ± 2,9efgA	78.0 ± 2.8cdeA	Callus formed	Callus formed
2000	20	60.7 ± 3.4hiA	70.7 ± 3.1efgB	Callus formed	Callus formed

Each value is the mean ± SE of 30 replications.
Values followed by the small different letters within columns or capital letters within rows are significantly different at $p<0.05$.
Medium I :3/2 MS containing 10 mg l^{-1} riboflavin ; Medium II: 3/2 MS containing 500 mg l^{-1} activated charcoal

Discussion

It has been reported that the major problem in tissue culture of papaya from mature field-grown plants are the endogenous bacteria excreted from the cut surface of the explants that grow throughout the media (Litz & Conover, 1978a, 1978b, 1981; Drew & Smith, 1986; Rajeevan & Pandey, 1986; De Winnaar, 1988; Mondal *et al.*, 1990; Reuveni *et al.*, 1990; Thomas *et al.*, 2007; Thomas & Kumari, 2011). We also experienced similar difficulties; only 8.3% contamination-free explants were obtained while culturing buds of mature field-grown hermaphroditic cv. Meizhonghong plants *In vitro* with conventional disinfection. After seven days of spraying with 1000 mg l^{-1} Gentamycin or 600 mg l^{-1} RIF before removing the explants and shaking them with 500 mg l^{-1} Gentamycin or 300 mg l^{-1} RIF, we found few buds contaminated from either greenhouse- or field-grown plants. This result differs from Reuveni *et al.*, (1990) who reported that antibiotic treatment was inefficient for buds from field-grown plants. However, the highest percentage of contamination-free explants achieved was only 55% from mature plants. From seedlings, 81.7% of explants were contamination-free with 0.1% $AgNO_3$ treatment after conventional disinfection, which was higher than with antibiotic treatments. We infer that shoot bud explants from seedlings were not seriously contaminated by endogenous bacteria. When seedling sex was determined, we multiplied hermaphrodite cultures from seedling explants kept in subculture at 15°C to slow multiplication and growth, as well as from cultures at normal temperature.

The initial response of cv. Meizhonghong to culture was within 8 d from seedlings and 12 d from fruit-bearing plants on MS medium supplemented with 0.5 mg l^{-1} BA. After four weeks, the explants were transferred to the same medium, and a number of adventitious buds regenerated. GA_3 and kinetin were not needed to break bud dormancy and promote stem elongation and shorten establishment time as previously reported (Litz & Conover, 1978a, 1978b**;** Rajeevan & Pandey, 1986; Mondal *et al.*, 1990). The difference may be related to the different genotypes of plant used (Jabeen *et al*., 2005). In our study, shoots derived from seedlings produced roots and adventitious shoots more easily than adult trees, consistent with previous reports (De Fossard *et al.*, 1977; Drew & Smith, 1986; Drew, 1988). In our experiments, explants from seedling were more effective than from greenhouse and field fruit-bearing plants in a mass propagation of the cultivars, despite waiting six months to determine sex. Javed *et al*., (2012) also reported tissue culture of plants were uaually affected different physiological state of explants. There was a stable average of 6.7-fold proliferation rate per subculture on MS media containing 0.25 mg l^{-1} BA after five subcultures at four-week intervals, the multiplication rate gradually increased from two to four subcultures. High BA concentrations caused excessive callus production, the optimal concentrations of BA (0.25–0.5 mg l^{-1}) at the multiplication stage (with low callus production) were similar to previous reports (Drew & Smith, 1986; De Winnaar, 1988; Reuveni *et al.*, 1990). Litz & Conover (1981) failed to re-establish apical dominance in shoots after 12-13 subcultures and Yie & Liaw (1977) reported the ability to regenerate was lost after eight successive subcultures. In contrast, shoot culture has been maintained by subculturing without any loss in the

multiplication rate or change in growth habit (Litz & Conover, 1978a, 1978b; Rajeevan & Pandey, 1986; De Winnaar, 1988; Mondal *et al.*, 1990), consistent with our result for subculturing where the culture was maintained for > 20 months (22 subcultures) without any decline in multiplication rate or change in growth habit.

The GA_3 can induce elongation of papaya shoots and other plant (Ouzounidou *et al.*, 2008) *In vitro*, but decrease the shoot proliferation rate (De Winnaar, 1988). We obtained a 4.20-fold proliferation rate, and an average height of shoots of 1.54 cm, which is suitable for rooting on 3/2 MS basal medium containing 0.25 mg l^{-1} BA, 0.3 mg l^{-1} GA_3 and 50 g l^{-1} sucrose in subcultures. We can also use this medium for subculture of adventitious bud proliferation in mass production because of the ease of manual transfer.

It is very difficult to produce high rooting percentages and high-quality adventitious root systems, needed to ensure establishment of plants in soil, from adventitious buds of *C. papaya* on a medium (De Winnaar, 1988; Drew & Miller, 1989). There are a number of reports concerning rooting of papaya (Drew, 1987; Drew & Miller, 1989; Drew *et al.*, 1991, 1993; Yu *et al.*, 2000). IBA has been found to be efficient for root induction (Drew, 1987; De Winnaar, 1988; Reuveni *et al.*, 1990) but the roots produced were thick and stumpy. In *C. papaya* cv. Honey Dew, comparatively high IBA concentration of 2.0 mg l^{-1} induced profuse rooting within 15-45 d of culture without producing any callus (Mondal *et al.*, 1990); however, in our study, in this media, there were lots of calli and roots were stout. Plant quality improved when shoots were transferred to hormone-free medium containing 10 µmol l^{-1} riboflavin injected after 2 d or 300 µmol l^{-1} riboflavin after 1 d or 500 µmol l^{-1} riboflavin after 2 d (Drew *et al.*, 1991, 1993). Because of the practical difficulty and cost of transferring shoots to media or injecting riboflavin into media in mass micropropagation, the beneficial effects of short dipping with IBA can be achieved cheaply and easily. Shoots dipped and transferred to a media containing activated charcoal had higher rooting percentages and better adventitious root systems than those treated with riboflavin.

There is no report of the use of activated charcoal for root induction and development culture of papaya, possibly because the usual concentration of activated charcoal (250–1000 mg l^{-1}) in media inhibits intensive root induction. In our study, the content of activated charcoal greatly influenced root induction and development. Without pretrearment or preculture, when activated charcoal > 250 mg l^{-1} in media, the regeneration rate was very low, while activated charcoal < 60 mg l^{-1} produced short and thick roots or only produced calli, with pretrearment or preculture, activated charcoal of 500 mg l^{-1} was most suitable for both high rooting percentage and good root quality in root development culture, possibly since activated charcoal can absorb certain auxins and improve aeration (Ernst, 1974; Dumas & Monteuuis, 1995; Vijaya Chitra & Padmaja, 1999).

The poor survival of papaya plants when transferred to soil limits the success of propagation (De Winnaar, 1988). In our study, 87% of plantlets survived in bags with sand (1/3 v) over the mixture of sieved peat and vermiculite (1:1 v/v), which was better than in the sand, sieved peat and vermiculite mixture in the greenhouse. It may be that the sand over mixture contained less water, whereas the mixture of sieved peat and vermiculite can maintain high relative humidity and avoid rotting of the root after transplanting. To our knowledge, there have been no reports of using this method to transplant *In vitro* papaya plantlets. About 100,000 plants were produced and planted successfully in the field within three years. Compared to mother plants, the *In vitro* raised plants did not show poor phenotypes, and all plants were hermaphrodites. Our findings are similar to the reports of Reuveni *et al.,* (1990) and Mondal *et al.,* (1990) and other plant (Khan *et al*., 2006) in this respect.

Commercial *In vitro* mass propagation has not yet been reported because of various problems during micropropagation of papaya that induce microbial contamination of explants, low proliferation rate and short multiplication clusters in multiplication, stout and thick roots of *In vitro* plantlets and low survival rate of transplanted plantlets. *In vitro* regeneration of papaya was systematically studied in this paper and it was concluded that *In vitro* regeneration system using apices and axillary buds from seedlings or fruit-bearing plants in the greenhouse is suitable for mass multiplication of the hermaphrodite *C. papaya* cv. Meizhonghong.

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